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16. ABSTRACT

Acute ethanol toxicity was evaluated in genetically defined mice by employing the conditioned-flavor-aversion paradigm. Mice of the C57BL/6 and BALB/c strains, and the F_1 cross between C57BL/6 males and BALB/c females, were accustomed to consume water during daily 4-hour periods of availability. Saccharin solution (0.1% w/v) was then substituted for water and 4 hours later each mouse received ethanol (0, 1.5, 3.0, 4.5 or 6.0 g/kg b.wt) via gastric intubation. Beginning one week later, saccharin intake was measured during 24-hour periods of availability at weekly intervals for 4 weeks. C57BL/6 and F_1 mice that had received ethanol consumed less saccharin than respective control mice that had not received ethanol. The decrease in saccharin intake was generally related to the dose and gradually diminished over subsequent exposures to saccharin without ethanol. Saccharin intake in BALB/c mice that had received ethanol did not differ from the intake of control mice. These data suggest that genetic variables may determine the extent to which toxicant-induced conditioned flavor aversions can be established in mice.

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ETHANOL-INDUCED FLAVOR AVERSIONS IN MICE A BEHAVIOR-GENETIC ANALYSIS[1,2]

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Acute ethanol toxicity was evaluated in genetically defined mice by employing the conditioned-flavor-aversion paradigm. Mice of the C57BL/6 and BALB/c strains, and the F_1 cross between C57BL/6 males and BALB/c females, were accustomed to consume water during daily 4-hour periods of availability. Saccharin solution (0.1% w/v) was then substituted for water and 4 hours later each mouse received ethanol (0, 1.5, 3.0, 4.5 or 6.0 g/kg b.wt.) via gastric intubation. Beginning one week later, saccharin intake was measured during 24-hour periods of availability at weekly intervals for 4 weeks. C57BL/6 and F_1 mice that had received ethanol consumed less saccharin than respective control mice that had not received ethanol. The decrease in saccharin intake was generally related to the dose and gradually diminished over subsequent exposures to saccharin without ethanol. Saccharin intake in BALB/c mice that had received ethanol did not differ from the intake of control mice. These data suggest that genetic variables may determine the extent to which toxicant-induced conditioned flavor aversions can be established in mice.

INTRODUCTION

Genetically defined (isogenic) strains of mice have been widely used for evaluating genetic determinants of the behavioral effects of drugs and other toxic substances (Anisman, 1975; Brase *et al.*, 1977; Festing, 1979; Shuster *et*

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[1]This research was conducted during the authors' tenure with the Neuroscience Program at NCTR. The opinions expressed herein are those of the authors and should not be construed as reflecting official policy.

[2]A preliminary report of these data was presented at the annual meeting of The Federation of American Societies for Experimental Biology, April, 1979 (Fed. Proc. 38:379, 1979).

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al., 1975; Broadhurst, 1977). As an illustration, a large body of literature has shown that the behavioral actions of ethanol differ markedly between inbred strains of mice (McClearn, 1968, 1979; Fuller, 1964; Goldstein and Kakihana, 1975; Goodrick, 1978). Mice of the BALB/c strain display a loss of the righting reflex for longer durations following large doses of ethanol than do C57BL/6 mice (McClearn, 1962; Kakihana *et al.*, 1966; Randall and Lester, 1974). Following an exposure regimen to ethanol vapor designed to induce physical dependence (Goldstein, 1972), BALB/c mice display greater and longer-lasting withdrawal seizures than do C57BL/6 mice (Goldstein and Kakihana, 1975). Moreover, when confronted with a choice between water and a solution of ethanol, BALB/c mice routinely choose water whereas C57BL/6 mice routinely choose ethanol (McClearn and Rodgers, 1959, 1961; Fuller, 1964; Goodrick, 1978; Wood, 1976).

Isogenic hybrid strains have also been used in evaluating the heritability of a particular trait, including the degree to which toxicants affect behavior. Studies which have compared the behavioral effects of drugs in the offspring of a cross between C57BL mice and mice of the BALB/c or DBA strains have typically shown that the effects in the offspring are either intermediate or resemble more closely those seen in the C57BL parent strain (Fuller, 1964; McClearn and Rodgers, 1961; Castellano and Oliverio, 1975; Oliverio *et al.*, 1973; Goodrick, 1978). For example, Fuller (1964) and McClearn and Rodgers (1961) obtained an intermediate inheritance for ethanol preference in offspring of the cross between C57BL and BALB/c or DBA mice. On the other hand, Oliverio and his coworkers (Castellano and Oliverio, 1975; Oliverio *et al.*, 1973) have shown that the same hybrids are very similar to the C57BL parent strain in terms of the effects of amphetamine, scopolamine and morphine on locomotor activity (see also De Fries and Hegmann, 1970; Messeri *et al.*, 1972).

A particularly useful procedure for assessing the acute toxicity of drugs and other chemicals is the conditioned-flavor-aversion paradigm. In this procedure, the test chemical is administered immediately after the animal has ingested a substance with a novel flavor, for example an aqueous solution of saccharin. After a period of several days during which any immediate effects of the test chemical dissipate, the animal is tested for consumption of the novel flavor solution which was paired with the chemical. Typically, smooth inverse dose-effect relations are found relating the amount of the novel flavor solution consumed to the dosage of the test chemical (Garcia *et al.*, 1974; Revusky and Garcia, 1970). Conditioned flavor aversions, moreover, represent a mode of learning that is seen in a wide range of vertebrate and invertebrate speices, and that can be induced by most of the psycho-active drugs and common poisons (Riley and Baril, 1976; Logue, 1979). To date, however, there have been comparatively few demonstrations of conditioned flavor aversions in mice, and very few attempts to evaluate genetic differences in the ability to form conditioned flavor aversions (Dudek and Fuller, 1978; Horowitz and Whitney, 1975). The present experiment was designed, therefore, to determine the effectiveness of ethanol in inducing conditioned flavor aversions in two inbred strains of mice and their hybrid cross.

METHODS

Animals

Fifty male retired breeders from each of the following strains served: C57BL/6J$_f$C3H$_f$/Nctr (C57BL/6), BALB/c StCrl$_f$C3H$_f$/Nctr (BALB/c) and B6CF$_1$ (the F$_1$ cross between C57BL/6 females and BALB/c males)[1]. The approximate ages and body weights (±1 SD) of each strain at the start of the experiment were: for C57BL/6, 16-24 wks, 32.0 ± 2.55 g; for BALB/c, 22 wks, 32.2 ± 2.60 g; and for F$_1$, 20 wks, 32.7 ± 2.14 g. Mice were housed individually in ceiling suspended cages with wire mesh floors (Hoeltge, Inc., Cincinnati, OH) located in a large temperature- and humidity-controlled colony room that operated on a 12-hr-on, 12-hr-off lighting cycle that began at 0600 hr each day. Except as detailed below, each mouse had unlimited access to autoclaved-Purina 5010C pellets, and Millipore-filtered autoclaved water.

Procedure

Upon placement in the colony room, the mice were adapted to handling and then to a schedule of water availability for four hours daily beginning at 0900 hr. Water was provided in autoclaved glass bottles (150 ml) fitted with rubber stoppers and bent metal drinking spouts. Water intakes were determined by weighing the water bottle and cage, without mouse or food dispenser, before and after each period of availability. This procedure was in effect for 4 days. On the fifth day, a 0.1% (w/v) solution of sodium saccharin was made available instead of water during the 4-hour drinking period. Within five minutes after termination of saccharin availability, each mouse received ethanol intragastrically in dosages of 0.0 (water vehicle control), 1.5, 3.0, 4.5 or 6.0 g/kg b.wt.[2] Injection volume was 0.01 ml/g b.wt. Ten mice of each strain were assigned to each dosage condition. Following ethanol, water was made freely available for the duration of the experiment. At weekly intervals for four weeks, however, saccharin was substituted for water, and intake was measured over a 24-hr period.

For each strain of mice the extent of the ethanol-induced conditioned flavor aversion was assessed by comparing saccharin intake in vehicle-control mice, which were not exposed to ethanol, with that of the mice receiving saccharin and ethanol. For vehicle-control mice, the effect of saccharin on intake was assessed by comparing saccharin intake with the intake of water on the preceding day.

RESULTS

Intakes in vehicle-treated mice

Mice of each strain had approximately the same intake when water was available for four hours daily. Average water intakes (±1SE) for the vehicle-

[1]In conducting this research, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

[2]We gratefully acknowledge the expert assistance of Ms. J. Buelke-Sam in dosing the mice.

control groups on the day prior to the first exposure to saccharin were 3.3 (±0.2), 3.3 (±0.1), and 3.2 (±0.2) ml for C57BL/6, BALB/c and F_1 mice, respectively. Similar intakes were also obtained for all strains on the first exposure to saccharin (3.7 ± 0.3, 3.4 ± 0.2 and 3.6 ± 0.2 ml, respectively). Strain differences in intake did emerge, however, during subsequent testing when fluids were available for 24-hr periods (see Table 1). On the days that preceded each 24-hr exposure to saccharin, C57BL/6 mice consistently consumed approximately 13% more water than BALB/c mice and approximately 22% more water than F_1 mice. The percentage increase in intake when saccharin was available was comparable for C57BL/6 and F_1 mice (88 ± 10% and 78 ± 9%, respectively), and was considerably greater than that for BALB/c mice (21 ± 5%).

Intakes in ethanol-treated mice

Mice of the C57BL/6 and F_1 strains that had received saccharin followed by ethanol consumed less saccharin than vehicle-control mice when it was next presented (Figure 1). The decrease in saccharin intake was generally dose-related and approximately equal in magnitude for the two strains, as can

TABLE 1. Water and saccharin intakes for vehicle-control mice. Each value represents mean intake ± SE in ml of 10 mice during 24-hr periods of availability. Values at the far right represent saccharin intake as a ratio to water intake on the preceding day.

Exposure Period	Water	Sacch	Sacch / Water
1			
C57	6.9 ± 0.4	12.8 ± 0.8	2.03 ± 0.08
BALB	5.3 ± 0.4	6.9 ± 0.3	1.34 ± 0.08
F_1	5.3 ± 0.3	9.5 ± 0.6	1.81 ± 0.12
2			
C57	6.8 ± 0.4	13.0 ± 0.9	1.90 ± 0.10
BALB	6.0 ± 0.6	7.1 ± 0.5	1.19 ± 0.06
F_1	5.3 ± 0.4	10.3 ± 0.4	1.99 ± 0.12
3			
C57	7.3 ± 0.3	11.6 ± 0.7	1.59 ± 0.08
BALB	6.6 ± 0.6	6.8 ± 0.4	1.17 ± 0.07
F_1	6.1 ± 0.2	9.3 ± 0.5	1.53 ± 0.10
4			
C57	6.1 ± 0.5	12.2 ± 0.7	2.00 ± 0.07
BALB	6.1 ± 0.4	6.7 ± 0.4	1.13 ± 0.04
F_1	5.7 ± 0.3	10.0 ± 0.8	1.77 ± 0.12
$\bar{X}$			
C57	6.8 ± 0.4	12.4 ± 0.8	1.88 ± 0.10
BALB	6.0 ± 0.5	6.9 ± 0.4	1.21 ± 0.05
F_1	5.6 ± 0.3	9.8 ± 0.6	1.78 ± 0.09

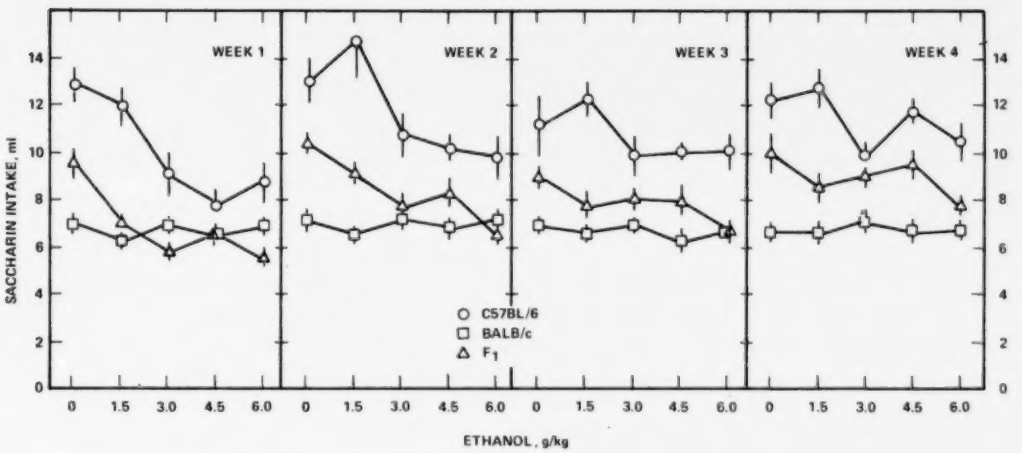

FIGURE 1. Effect of ethanol on the saccharin intake of mice. Ethanol was given following the initial exposure of the mice to saccharin. Each symbol represents the mean effect of ethanol, and ethanol vehicle, on the 24-hr saccharin intake (in ml) of 10 mice of the C57BL/6 (○), BALB/c (□) and F_1 (△) strains when saccharin was provided at weekly intervals after the saccharin-ethanol pairing. Vertical bars represent ± 1 SE.

be seen in the left-most panel of Figure 1. An estimate of the statistical reliability of ethanol's effects can be made in the following way. The effect of a given dose was considered to be significant at the 0.01 level when there was no overlap between the standard error of the mean for that dose and the limit defined by three standard errors of the mean for the appropriate vehicle-control group. Significant decreases in saccharin intake were thus obtained at 3.0 g/kg and greater for C57BL/6 mice and at 1.5 g/kg and greater for the F_1 mice. Mice of the BALB/c strain that had received ethanol, however, showed no decrease in saccharin intake when compared with those that had not received ethanol. Continued exposure to saccharin diminished the initial ethanol-induced decrease in saccharin intake in C57BL/6 and F_1 mice, as can be seen in the succeeding panels of Figure 1. Note, however, that saccharin intake in BALB/c mice that had received ethanol did not differ at any time from the intake of BALB/c mice that had not received ethanol.

Relationship Between Baseline Saccharin Intake and Ethanol-Induced Decreases in Intake

The possible contribution of differences in saccharin intake to the intake-decreasing effect of ethanol was assessed in the following manner. Scattergrams were constructed of the logarithm of the mean saccharin intake for each strain that received ethanol *versus* the logarithm of the mean saccharin intake for the corresponding group that did not receive ethanol. Figure 2 presents the results for the 4.5-g/kg dosage for the first and fourth 24-hr period in which saccharin was available. The data are reasonably well described by a linear function, suggesting that the decreases in saccharin intake produced by

ethanol were a power function of the amount of saccharin consumed in the absence of subsequent ethanol. Although not shown, equally good fits were obtained for the other ethanol groups. Moreover, additional exposures to saccharin without ethanol produced systematic increases in the slope of the functions, and by the fourth week the functions did not differ from the dashed no-effect line shown in Figure 2.

DISCUSSION

Ethanol induced prominent aversions to saccharin in mice of the C57BL/6 strain and the cross between C57BL/6 females and BALB/c males, but did not induce a saccharin aversion in those of the BALB/c strain. These results support earlier work that demonstrated conditioned flavor aversions in mice (Etscorn, 1973; Horowitz and Whitney, 1975) and in addition draw attention to genetic variables as potential determinants of the extent to which conditioned flavor aversions can be established. These data further suggest that caution must be exercised in interpreting the results of mouse experiments employing the conditioned-flavor-aversion paradigm as an assay for acute behavioral toxicity, since a negative result may be more a function of the strain selected than the chemical in question (Festing, 1979).

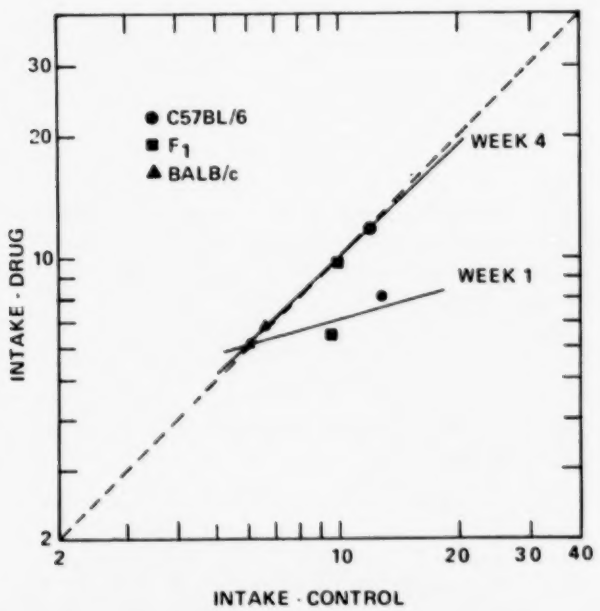

FIGURE 2. Relationship between saccharin intake of control mice and that of mice that had received ethanol (4.5 g/kg). These data are replotted on logarithmic coordinates from those portrayed in the left-most and right-most panels of Figure 1.

The present experiment employed C57BL/6 and BALB/c inbred mice because a wealth of data has shown that the two strains differ substantially with respect to ethanol's behavioral effects. For example, BALB/c mice display greater impairments in righting, and longer, more intense withdrawal seizures than do C57BL/6 mice following large dosages of ethanol (McClearn, 1962, 1968; Kakihana *et al.*, 1966; Randall and Lester, 1974; Goldstein and Kakihana, 1975). Several experiments have also shown that C57BL/6 mice prefer ethanol solutions over water whereas the reverse is generally true for BALB/c mice (McClearn and Rodgers, 1959; Fuller, 1964; Goodrick, 1978; Wood, 1976). These differential effects of ethanol between BALB/c and C57BL/6 mice, and similar differences in effect between DBA/2 and C57BL/6 mice, have been interpreted as a reflection of differences in enzyme activities involved in the metabolism of ethanol. In this regard, Sheppard *et al.* (1968) found slightly greater hepatic alcohol dehydrogenase activity, and substantially greater aldehyde dehydrogenase activity in C57BL/6 than in DBA/2 mice. In addition, DBA/2 mice had substantially greater blood levels of the toxic metabolite acetaldehyde after ethanol than did C57BL/6 mice (Sheppard *et al.*, 1970). It is important to note, however, that actual *in vivo* rates of the metabolism of ethanol are comparable in C5BL/6 and DBA/2 as well as BALB/c mice (Rodgers, 1966, 1972; Kakihana *et al.*, 1966). Nevertheless, on the basis of these data, one might predict that ethanol would induce a greater and more long-lasting flavor aversion in BALB/c and DBA/2 mice than in C57BL/6 mice, and the data of Horowitz and Whitney (1975) support such a prediction for DBA/2 and C57BL/6 mice. In the present experiment, however, ethanol induced a substantial flavor aversion in C57BL/6 mice but was ineffective in BALB/c mice, indicating a dissociation between ethanol's ability to induce flavor aversions and its pattern of metabolism in these two strains (McClearn, 1979; Belknap *et al.*, 1972; Randall and Lester, 1974; Rodgers, 1966, 1972). Although these results are at variance with those obtained by Horowitz and Whitney (1975), numerous procedural differences preclude at this time a precise delineation of the source(s) of this variance. The data do, however, highlight the importance, when assessing genetic differences in the behavioral effects of ethanol and other toxic substances, of studying a wide array of classes of behavior.

The finding that ethanol-induced aversions to saccharin occurred in both the C57BL/6 and the F_1 mice suggests that the ability of ethanol to induce flavor aversions is preferentially inherited. These results are in support of data derived from cross-breeding experiments in which behavioral features of the offspring of a cross between C57BL and BALB/c mice, or between C57BL and DBA mice, resemble more closely the C57BL/6 than the other parent strain (Castellano and Oliverio, 1975; De Fries and Hegmann, 1970; Messeri *et al.*, 1972; Oliverio *et al.*, 1973). The present data offer only tentative support for this conclusion, however, since the F_1 mice were derived from cross-breeding C57BL/6 females with BALB/c males. Assessment of the role of prenatal environmental factors in ethanol-induced flavor aversions would require the use of reciprocally crossed hybrids (McClearn and Rodgers, 1961) or ova transfers (Randall and Lester, 1975; Broadhurst, 1977). The present experiment

demonstrates, nevertheless, the benefits of utilizing several isogenic strains in the comprehensive assessment of the acute toxicity of manufactured chemicals (Festing, 1979).

The possibility exists that the obtained strain differences in the extent to which ethanol produced flavor aversions depended primarily on differences in the animals' responses to saccharin in the absence of ethanol. Table 1 shows that while the vehicle-control groups did not differ materially in water intake, there were appreciable differences in saccharin intake, with C57BL/6 and F_1 mice consuming considerably more than BALB/c mice. Moreover, mice of all strains consumed more fluid on days when saccharin was available than when water was available. For BALB/c mice, the increase was small, while for C57BL/6 mice intake was almost doubled when saccharin was the only available fluid. Figure 1 shows that the effect of ethanol was proportional to this saccharin-induced increase in fluid intake and consisted of a decrease in the amount of excess fluid consumed on saccharin days. In no case, however, was fluid intake reduced below the normal level of water intake. In fact, from the flattened dose-effect functions at large doses of ethanol, it appears that the aversion to saccharin was not sufficiently strong to overcome the animals' normal requirements for water. This suggests that a two-bottle test, in which saccharin and water are concurrently available during the test period, might be a more sensitive method for the detection of toxicant-induced flavor aversions (Dragoin *et al.*, 1971; Elkins, 1973).

Another possibility is that differences in the extent to which ethanol produced conditioned flavor aversions were due directly to differences in baseline saccharin intake, and only indirectly due to strain differences. This possibility is supported by the orderly relations that were obtained between the saccharin intakes of ethanol-treated mice and those of the appropriate vehicle-control mice (Figure 2). In many experimental contexts, differences in non-drug control baselines have been shown to be powerful determinants of the effects of many drugs (Dews and Wenger, 1977; MacPhail and Gollub, 1975; Irwin *et al.*,1959; Wilder, 1967). An example of the importance of baseline features in behavior-genetic research is provided by McClearn and Shern (1975) in which strain-related differences in the effect of ethanol on open-field activity were confounded by differences in baseline activity levels in the absence of ethanol. It is important, therefore, that the study of genetic determinants of the behavioral effects of drugs and other toxicants be carried out, insofar as possible, in the absence of underlying differences in baseline levels of behavior (Wenger, 1979).

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